In Vitro *Inhibition of Human Sarcoma Cells' Invasive Ability by Bis(5-amidino-2-benzimidazolyl)methane — A Novel Esteroprotease Inhibitor*

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Bis(5-amidino-2-benzimidazolyl)methane (BABIM) is a synthetic aromatic amidine compound which has a number of important biochemical effects, including inhibition of a family of esteroproteases (trypsin, urokinase, plasmin) previously linked to the complex process of tumor invasion. Previous work has suggested that exogenous natural protease inhibitors can block invasion of tumor cells across basement membranes (BM) *in vitro*. The authors studied the effect of BABIM on the human cell line HT-1080 with the use of a quantitative *in vitro* amnion invasion assay system. They have verified the ability of these cells to grow in nude mice and metastasize via the lymphatics or blood vessels on the basis of the route of administration of the inoculum. Cells which were able to actively cross the entire BM were trapped on filters and counted by both brightfield microscopy and by beta scintillation counting of cells whose DNA was labeled with tritiated thymidine. In agreement with either counting technique, BABIM, at a concentration of 10^{-4} M, significantly inhibited invasion ($P < 0.005$) over the 7-day course of the experiments. Under these conditions, the inhibitor was nontoxic and did not alter the attachment of the cells to the amniotic membrane. Furthermore, a highly significant inhibition of invasion ($P < 0.001$) was also demonstrated across a variation in molar concentration of BABIM of more than 2 orders of magnitude. Most remarkably, cells were initially inhibited in their ability to invade in the presence of between 10^{-9} and 10^{-3} M BABIM. Measurement of Type IV specific collagenase in media from these cells shows a significant inhibition of activity in the presence of BABIM. These results suggest two, not necessarily exclusive, alternative interpretations: first, that inhibition of the proteolytic steps along the pathway of activation of basement membrane degrading enzymes results in inhibition of invasion; second, that arginine directed esteroproteases may work in concert with cellular collagenolytic metalloproteinases in the process of invasion by human tumor cells through native matrix barriers. (Am J Pathol 1986, 123:46–56)

IN ORDER for metastases to occur, tumor cells must have the capacity to invade normal tissues, especially capillary and lymphatic vascular spaces.[1] Basement membranes are a major barrier to metastatic spread, because tumor cells must often invade across epithelial/dermal basement membranes as well as vascular basement membranes in both directions.[2] Enzymatic proteins and proteases, especially those intimately associated with the coagulation system, including plasmin and plasminogen activator, have been strongly linked to cell invasion (by both benign and malignant cells) through extracellular matrices.[3-8] Antibodies directed against plasminogen activator have been shown to inhibit human tumor metastasis from carcinomas.[9] Plasminogen activator has recently been used as a marker for neoplastic transformation in studies including those utilizing flow cytometry,[10,11] although with mixed results.[12]

Natural collagenase inhibitors exist in higher organ-

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isms, including man and are found in the serum as well as in the extracellular fluid.[13-15] Furthermore, natural inhibitors applied exogenously to experimental systems have been shown to inhibit tumor cell invasion.[16] Thorgeirsson et al have demonstrated a "450% decrease" in invasion of malignant mouse reticulum cell sarcoma cells by exposing the cells to natural inhibitors of both serine proteinases and metalloproteinases.[17] Other experimental studies have yielded similar results.[18-21] This raises the hope that a variety of protease inhibitors may block tumor invasion.

We have previously synthesized a series of aromatic mono- and diamidines which may work in part through a similar antiprotease mechanism.[22,23] Twelve synthetic amidino compounds were screened for their ability to inhibit tumor invasion *in vitro* (unpublished results). Using one of these, we have performed a series of experiments testing the ability of a synthetic arginine-directed esteroprotease inhibitor, bis(5-amidino-2-benzimidazolyl)methane (BABIM), to inhibit the invasion of HT-1080 human fibrosarcoma cells through living human basement membranes, using an amnion *in vitro* model system of invasion.[24] Once tumor cells penetrate the basement membrane in this model, they become lodged in an underlying inert methyl cellulose filter. In previous studies using this model, cells completely crossing the basement membrane and lodging in the filters were stained and counted with the use of standard brightfield microscopy.[25,26] Similar visual examination of the traversed human fibrosarcoma cells was performed in this study, but in addition, a long-term tritiated thymidine cell labeling technique was utilized which proved to be easier to perform and gave more reproducible results. These studies were repeated after the cells had been passaged through BALB/c/NIH Swiss hybrid mice.

To verify that the inhibitory effects of BABIM were due to inhibition of invasion, and not secondary to either decreased cellular proliferation or altered attachment to matrix, the kinetics of cell growth in the presence and absence of BABIM was also studied. Finally, as a direct test of BABIM's ability to inhibit one of the proteolytic enzymes linked to invasion, we examined the ability of BABIM to alter the cell's production of an enzyme which specifically cleaves Type IV (basement membrane) collagen.

Materials and Methods

Cell Culture

Human fibrosarcoma cells (HT-1080), originally derived from a primary human acetabular bone tumor,[27] were a gift from Dr. Judson J. Van Wyk, Department of Pediatrics, University of North Carolina at Chapel Hill. They were originally obtained from the Cell Culture Laboratory, University of California, Naval Biosciences Laboratory, Naval Supply Center, Oakland, California, and maintained in continuous culture at the tissue culture facility of the Lineberger Cancer Research Center, University of North Carolina at Chapel Hill. The cells were grown at 37 C in 5% CO_2/95% air in 100-mm tissue culture dishes (Falcon) with Dulbecco's minimal essential medium (DMEM) supplemented with 10% fetal bovine serum, 2 mM L-glutamine and penicillin G (100 μ/ml), streptomycin (100 μg/ml) and Fungizone (5 μg/ml) (all supplied by the Lineberger Cancer Research Center). This cell line contains the activated oncogene *N-ras* located on Chromosome 1,[28] produces tumors in nude mice, and has its tumorigenicity dependent upon chromosome dosage.[29]

Cell Proliferation Studies

To determine the effects of BABIM on cell growth, 1×10^5 HT-1080 cells were added to medium containing 50-mm tissue culture plates in the presence or absence of differing concentrations of BABIM. For one series of experiments, the cells were initially incubated in 10% fetal bovine serum-containing medium. This was exchanged for serum-free medium after the first day, thereby recapitulating the experimental conditions in the upper chamber of the amnion invasion assay chambers (as discussed below). Cell counts were performed in triplicate every 24 hours for 7 days.

Amnion Invasion Assay System

Lucite amnion invasion assay chambers were custom-made in the instrument shop of the Department of Physics and Astronomy, University of North Carolina, from a prototype provided by Dr. Lance A. Liotta, Laboratory of Pathology, National Cancer Institute.[30] The design of the chamber and methods pertaining to its use have been previously detailed by Russo et al.[31]

As modified by us, the amnion was prepared as follows: Blunt dissection was used to separate nonopaque viable amnion from routinely delivered placentas which were less than 18 hours old. The simple cuboidal epithelium was removed by alkali treatment in 0.1 N ammonium hydroxide. The orientation of the membrane was retained, and the amnion holders were fitted with the denuded side of the membrane up, which exposed the basement membrane to the HT-1080 cells and created an upper and lower chamber. A Millipore filter (0.45 μ) was placed beneath the membrane, in contact with the stromal side, to catch cells able to transverse the amnion.

Prepared chambers were placed in six-well tissue culture trays (Falcon or Costar) containing approximately 1.5 ml of serum-containing medium, which filled the bottom chamber. Under experimental conditions, serum-containing medium remained in the lower chamber throughout the experiment. At zero time, 5×10^5 harvested viable tumor cells were added to the upper chamber in the presence of 10% FCS-containing medium to enhance attachment of the cells to the amniotic basement membrane. Cell counts were performed in triplicate in a Coulter counter after the cells were washed in Hanks' balanced salt solution and exposed to 0.25% trypsinization. Previous trypan blue exclusion studies showed a viability greater than 97% when cells were prepared in this manner. After 24 hours, the medium in the upper chamber was removed and replaced with serum-free medium in the presence or absence of BABIM. Fresh BABIM-containing solutions were replenished every other day.

Quantitation of Invading Cells

In all experiments three of the six wells on any one tray contained medium with BABIM, and the other three did not contain BABIM. For each experiment each point of the time course was studied in triplicate. Invasion in amnion holder containing trays was stopped at Days 3, 5, and 7. One to two wells were prepared for each experiment without filters, and a drop of India ink was placed in the upper chamber so that we could evaluate and verify that the amnion was free of defects. Cells were measured for invasion by two different techniques: direct visual counting or ^{3}H-labeling of cells. In the former, the invasion was terminated at the appropriate times by fixation in 10% buffered formalin; the filters and amnion were stained with Mayer's hematoxylin, separated, placed on glass slides, and dehydrated with graded ethanol and xylene. Immersion oil was used to render the filters transparent. The filters/amnion were coverslipped and the slides examined at ×400 on a Leitz brightfield microscope with a mechanical stage. For the purpose of this study clusters of cells were counted as a single cell. The reproducibility and variability of this assay have been documented previously.[24]

In duplicate experiments, HT-1080 cells, in the log phase of growth, were long-term-labeled with methyl-tritiated thymidine (1 μCi/ml) (ICN) and left 24 hours before harvesting. At appropriate times the medium was removed and the filters were allowed to air-dry. After placement in a scintillation vial, the membranes were dissolved in 1 ml of 2-methoxyethanol, and to each vial 10 ml of scintillation cocktail was added (Scinti Verse-Fisher). The vials were vortexed, allowed to equilibrate in the dark, and counted in a Packard Tri-Carb beta scintillation counter. All counts were checked on a second scintillation counter, with each sample counted for 30 minutes for minimizing counting error. Statistical analysis of the experiments was performed using two-way analysis of variance and the Kruskal-Wallis one-way analysis of variance by ranks[32] as previously described.[26]

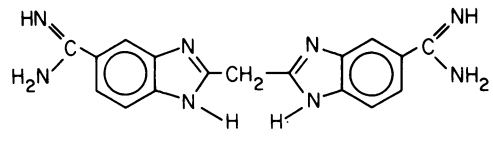
Protease Inhibitor

BABIM (Figure 1) is a synthetic low-molecular-weight aromatic amidine inhibitor of trypsin-like proteases.[22,33,34] The compound's purity has been verified both by nuclear magnetic resonance spectrometry and elemental analysis.[35] It has previously been shown to block respiratory syncytial virus-induced cytopathology[36,37] and inhibit plaque formation, as well as to reduce cell injury in *Rickettsia ricketsii* infections.[38] This compound has an inhibitory effect on trypsin (K_i = 0.017 ± 0.006 μM), urokinase (K_i = 2.33 ± 0.36 μM), thrombin (K_i = 4.15 ± 0.65 μM), and plasmin (K_i = 2.65 ± 0.47 μM), though this may not be the cause of its antimicrobial effect.[37]

In Vivo Experiments and Tumor Transplantation

Athymic (nude) virgin mice of the NIH I BALB/c/NIH Swiss hybrid (T-cell-deficient) strain[39] were bred and maintained in the Athymic Mouse Isolation Facility of the University of North Carolina at Chapel Hill. This facility meets or exceeds the legal standards of animal care and use established under federal laws and the policies on animal welfare published in the NIH guide for contracts and grants. Adult females were inoculated with varying doses of HT-1080 cells suspended in serum-free DMEM at a concentration of 1×10^6 cells/0.1 ml. We injected cells either subcutaneously into the right inguinal region to produce flank tumors and to measure "spontaneous" metastases or intrajugularly to quantitate "experimental" metastases. Animals receiving intrajugular injections were anesthetized with a 1:1 mixture of acepromazine maleate and ketamine hydrochloride at a concentration of 0.03 ml/30 g mouse for surgical exposure of the internal jugular vein. Six animals received flank or intravenous injections of vehicle only.

After 3 weeks, half of the tumor cell-injected animals were observed to have evidence of tumors and were humanely sacrificed along with an appropriate number of control animals. A gross necropsy was immediately performed, and selected tissues were removed and fixed in 10% buffered formalin for light-microscopic study. Tissues studied histologically included the right inguinal lymph node, flank tumor, and associated skin or



Bis(5-amidino-2-benzimidazolyl)methane
(BABIM)

Figure 1—The chemical structure of the synthetic low-molecular-weight aromatic amidine inhibitor BABIM. A coupling of the parent compound 5(6)-amidinobenzimidazole with itself across a carbon bridge resulted in a 100-fold increase in potency. In its natural state the compound will probably not be linear but rather will form hydrogen bonds between the N1 and N3′ nitrogen of the imidazole groups, transforming the compound into a 5-ring structure.

muscle if the tumor was fixed to tissue, liver, kidney, spleen, and heart/lung as one block. The lung was prefixed by an infusion of Bouin's fluid through the trachea prior to removal to aid in identification of metastatic foci. Excess Bouin's fluid was removed by multiple exchanges in 70% alcohol prior to embedding in paraffin. Remaining animals were allowed to survive and sacrificed as tumors became evident.

Tumor tissue from a primary flank mass having reached a size of approximately 2 × 1 × 1 cm was removed from a sacrificed animal. The tumor was minced into fragments <1 mm in size. They were placed in Petri dishes containing DMEM supplemented with 10% fetal bovine serum, and antibiotics and were allowed to grow out. The cells grew rapidly; and after 4 days cells, one to several layers thick, covered the dishes and were passaged. The resulting cells had a phenotypic appearance identical to the original HT-1080 cells and were then used in a repeat invasion assay in the presence or absence of 10^{-4} M BABIM.

Measurement of Type IV Collagenase

HT-1080 tumor cells (1×10^5) were suspended in serum-free medium containing antibiotics and seeded into triplicate wells for 72 hours in the presence or absence of 10^{-4} M BABIM. There was no qualitative difference in cell growth between the different groups. The secreted Type IV collagen-degrading metalloprotease activity was determined in an appropriate aliquot as described by Liotta et al,[5,20] as modified by us,[26] except that tritiated, rather than ^{14}C-proline-labeled Type IV collagen was used as substrate (a gift from Dr. T. Turpeenniemi-Hujanen). Latent enzyme was activated by 0.01% trypsin for 5 minutes at 37 C, and the reaction was assayed in the presence of a five fold-concentrated solution of soybean trypsin inhibitor and 3.8 mM N-ethylmaleimide. The reaction was terminated after 18 hours, by cooling to 4 C for 10 minutes with the addition of BSA, followed by trichloroacetic acid (10%)/tannic acid (0.5%) precipitation, in the presence of 2 mM L-proline, for 90 minutes at 4 C. The samples were centrifuged, and the supernatants were counted in a Packard B-scintillation counter. Bacterial collagenase (Sigma Chemical Company) was used as a positive control. Parallel control samples were treated with 15 mm EDTA to verify the metalloproteinase nature of the affected enzymes. The data are expressed as radioactivity (counts per minute) solubilized per 10^5 cells per 18 hours and as a percentage of the maximal degradation by bacterial collagenase in untreated controls which were fixed at 100%. Triplicates were performed, the experiment was repeated twice, and results were expressed as means ± the standard deviations. The results were subjected to the Student *t* test for comparison of means.[32] The linear range of the assay has previously been determined to be between 0% and 60% of the maximum substrate degraded by bacterial collagenase.[17]

Results

Invasion of HT-1080 Cells Through the Amnion

One hundred to five hundred thousand viable cells per invasion chamber were layered on the epithelial denuded amniotic basement membrane (BM) at the start of each experiment. Although tumor cells can invade across epithelial and endothelial cell-covered basement membranes in *in vitro* assays,[25] denuding the epithelial layer provided for a more reproducibly uniform surface and negated the potential problem of partial surface denudation during processing and handling as accounting for observed changes. During the first 24 hours the cells were in a DMEM supplemented with 10% fetal bovine serum to enhance attachment of the cells to the BM. At 24 hours the serum-containing medium was withdrawn from the cells and the experiments begun in serum-free medium. As was observed in other studies using this system, only a minor population of the total exposed cells were able to cross the membrane (<1%), become entrapped in the underlying filter, and remain viable for counting purposes (Figure 2 [inset]).[24,26] Of those cells eventually crossing the amnion, the majority (>60%) did so during the first 48 hours. The total number of cells that passed through varied greatly between experiments, thereby necessitating the use of a single amnion for any one set of experiments, the random selection of chambers to be divided into treated and nontreated groups, and performance of all experiments in triplicate. It was for these reasons that we chose to express our results below as percent inhibition rather than number of cells crossing onto the filter. Because the HT-1080 fibrosarcoma cells are fully transformed in culture, they do not show con-

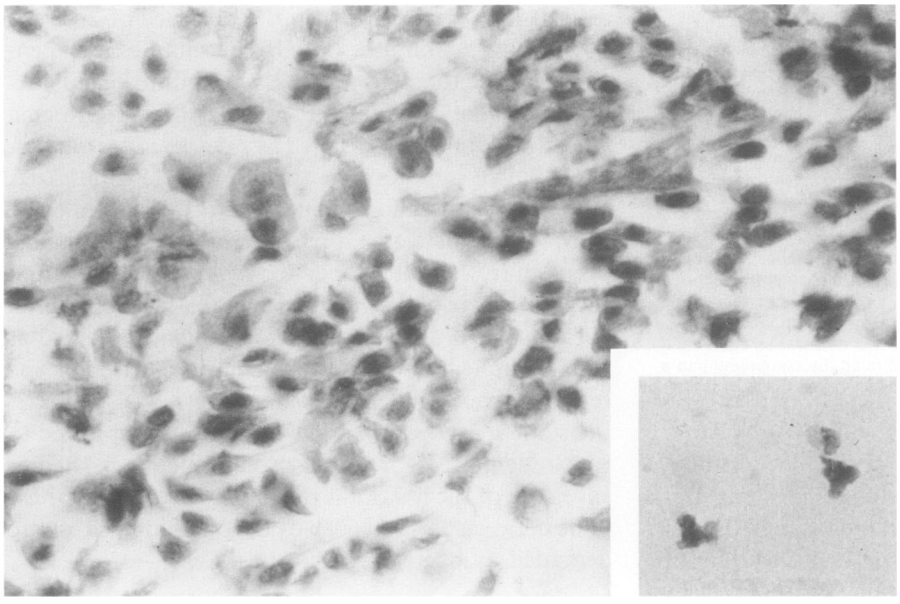


Figure 2—Photomicrograph of the mat of multilayered HT-1080 cells on the amnion basement membrane. The tumor cells were exposed to 10^{-4} M BABIM for 7 days in serum-free medium after an initial 24 hours in 10% fetal bovine serum-containing media to enhance attachment. (Hematoxylin, original magnification, ×250) **Insert**—Representative field of HT-1080 cells which successfully crossed the amnion and were trapped on the underlying methyl cellulose filter. (Hematoxylin, original magnification, ×250)

tact inhibition, but rather rapidly attach, cover the basement membrane-exposed surface, and proliferate, covering the surface with a multinumbered layer(s) of cells (Figure 2). This prevented accurate cell counts of tumor cell covered amnions. In all experiments reported the membrane was judged by scanning power (40×) viewing, to be covered in an equivalent manner in both experimental and control groups, or was excluded from the study.

For the first series of experiments, the effect of BABIM on *in vitro* invasion of HT-1080 cells was studied. These cells are one of a large number of mesenchymal-derived malignant cells successfully propagated in tissue culture which invade in the amnion system. BABIM has a molecular weight of approximately 300 and is a bicyclic amidino-substituted heterocyclic compound which strongly inhibits trypsin, plasmin, and urokinase-induced plasminogen activation.[22,35-38]

At Day 3 of exposure to a molar concentration of 10^{-4} BABIM, the cells demonstrated a mean inhibition of 27.8% as compared with the untreated controls. Cells were inhibited 46%, a highly significant finding ($P < 0.005$), when compared with untreated cells by Day 7 (185% inhibition by the method of Thorgeirsson[17]). This concentration of BABIM was initially selected because it has previously been shown to have a low degree of cytotoxicity in animal models in which it was studied.[35,38]

Because of the tedious nature (>2 hours/filter) of counting each Millipore filter at 400 diameters with a standard light microscope, we considered biosynthetically labeling the whole amninon with L-(U^{14}-C)-proline, in the presence of ascorbic acid and beta-aminopropionitrile in proline/glutamine-free DMEM supplemented with 20% dialyzed fetal calf serum, as has previously been suggested.[24] We found the variation of intensity of the label too great within a single amnion to be reproducible in our hands. Therefore, we elected to label the rapidly growing individual HT-1080 cell's DNA with ^{3}H-thymidine and measure the counts on the filter.

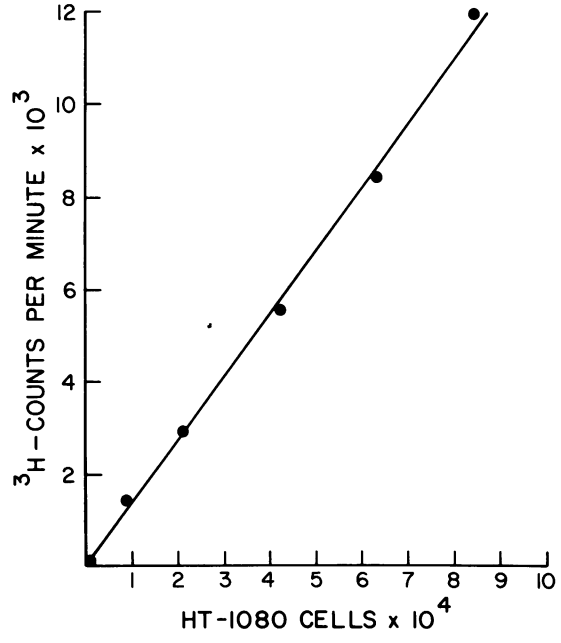


Figure 3—Demonstration of the linear relationship between tritiated counts per minute and numbers of cells on methyl cellulose filters. HT-1080 cells were grown for 24 hours in the presence of methyl-^{3}H-thymidine; and after trypsinization and washing, numbers of cells were counted and known amounts added to methyl cellulose filters. The points represent the mean of triplicate samples. The correlation coefficient of the line is 0.997.

Measuring Tritiated Thymidine-Labeled Cells

Figure 3 demonstrates the linear relationship which existed between the number of cells on the Millipore filter and the amount of radioactivity on the same filter. When duplicate experiments were performed with one set being counted microscopically and the other by scintillation counting for 30 minutes to minimize the standard error, the results were as shown in Figure 4. Within the range of experimental error, the percent inhibition of invasion was identical by both methods.

By this radiolabeling technique the maximum percent inhibition of invasion of viable tumor cells after 3 days of exposure to BABIM was between 59.1% and 76.4%, across a 2 orders of magnitude change in the molar concentration of BABIM in serum-free medium, as is demonstrated in Figure 5. At all three concentrations there was no difference in the cells' ability to attach to the BM and remain viable. However, unexpectedly, although the percent reduction of invasive cells increased by Day 7 in the cells treated with 10^{-4} M, the difference between the treated and control groups disappeared by Day 7 in the cells treated with either 10^{-3} or 10^{-5} M BABIM (data not shown). This does not negate the fact that, for example, in 10^{-3} M BABIM-treated cells, two-way analysis of variance demonstrated a time effect ($P < 0.005$), a treatment effect ($P < 0.005$), and a time treatment interaction ($P < 0.01$) for the treated versus nontreated cellular populations.

In order to assess the minimal effective inhibitory dose, cells were exposed to BABIM at a concentration from 10^{-3} to 10^{-24} M. At concentrations of 10^{-6} M or less there was no difference in inhibitory effect of BABIM on Day 5 or 7. However, at Day 3 there was still significant inhibition at concentrations diluted to between 10^{-9} and 10^{-10} M. A representative chart of inhibition between 10^{-3} M and 10^{-24} M is shown in Table 1.

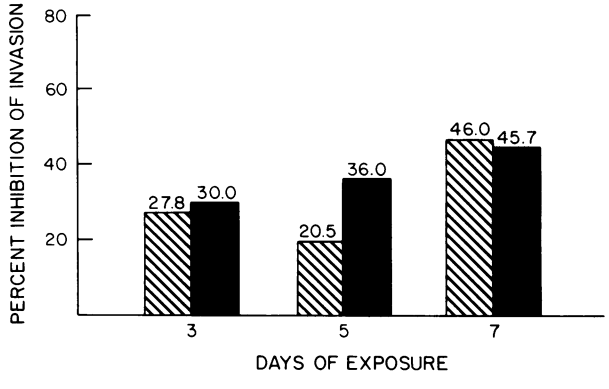


Figure 4—A histogram of the percent inhibition of invasion of HT-1080 cells in the presence of 10^{-4} M BABIM as compared with untreated cellular controls exposed to the identical amnionic membrane. BABIM-containing medium was replaced every other day. The *hatched boxes* represent the mean differences based on visual observation, and the *solid bars* are determinations based on beta scintillation counting. By the analysis of variance test for a two by three factorial experiment replicated three times, for the visually counted cells the results were highly significant (time effect, $P < 0.005$; treatment effect, $P < 0.005$; time/treatment interaction, $P < 0.025$).

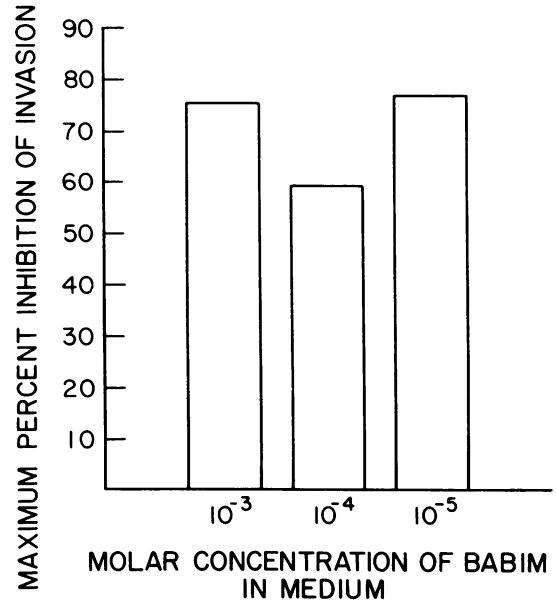


Figure 5—Maximal inhibition of invasion obtained after 3 days of exposure to serum-containing medium with from 10^{-3} to 10^{-5} M BABIM. For 10^{-5} M treatment as an example, the actual mean counts per minute were 736.6 for the control and 173.8 in the BABIM treated group. This corresponds to 5402 and 1275 cells, respectively. At each concentration tested the inhibition was highly significant.

Growth Kinetics of BABIM-Treated HT-1080 Cells

In order to exclude the possibility that the observed differences in treated and untreated cell populations were due to inhibition of cell growth in the presence of BABIM, growth curves of the cells were performed. As is seen in Figure 6A, multiple cultures of cells were plated at Day 0 in the presence of 10% fetal bovine serum, mimicking the conditions of the upper amnion invasion assay chamber. At Day 1 (arrow), the serum-containing medium was withdrawn, and the mean cell count, in the presence and absence of 10^{-4} M BABIM was quantitated. There was a small decrease in the total number of cells during the next 2 days, with the cells entering the log phase of growth between the fifth and seventh days. There was no significant difference during the early part of the experiment (for example, Day 3, where invasion inhibition was maximal). However, by Day 7, there was observed a 44% decrease in the cell count, closely paralleling the differences in invasive capability. Because the upper and lower chambers permit free diffusion of macromolecules, it was decided to repeat the cell growth curves in the presence of 10% fetal bovine serum (a normal constituent of the lower

Table 1—Effect of Varying Concentrations of BABIM on the Invasive Ability of HT-1080 Cells

Molar concentration of BABIM in media	% Inhibition of invasion*
10^{-4}	32
10^{-6}	29
10^{-9}	14
10^{-14}	0†
10^{-19}	0
10^{-24}	0

* Mean % inhibition (n = 4) of tumor invasion through amnion at 72 hours. See text for details.

† At very low concentrations some wells show a small stimulatory effect (<30%) as compared with treated cells, probably representing biologic variability in the system.

compartment). As is seen in Figure 6B, the cell kinetics across the seven days were now virtually identical. It thus appears that BABIM has no effect on cell growth of kinetics at the time when inhibition of invasion by HT-1080 cells is at its maximum.

In Vivo Experiments

Even though this line has been shown by others to grow in nude mice,[29] we repeated the experiments to verify the tumorigenic and metastatic nature of the cell line.[39] NIH-Swiss mice were treated with varying doses of HT-1080 cells by subcutaneous flank injection, for measurement of "spontaneous" metastasis and via intrajugular injection for measurement of "experimental metastasis."[40,41] Those mice receiving the largest dose by either route (2×10^6 cells) acquired local tumor growth and visceral metastases, including regional lymph node and adrenal gland involvement by the subcutaneous route and periarterial intrapulmonary metastases by the intravascular route, after 3 weeks (Figure 7). Additional animals continued to develop tumors and were sacrificed as the tumor burden became life-threatening. None of the controls showed spontaneous tumor development.

Cells from a primary flank mass were returned to tissue culture, passaged, and placed into amnion invasion assay chambers. An order of magnitude greater number of cells were able to cross the basement membrane than we have previously or subsequently seen in similarly run experiments. Quite unexpectedly, although the inhibition, by 10^{-4} M BABIM, at Day 3 was 52.5% (a figure not statistically different from that seen with the

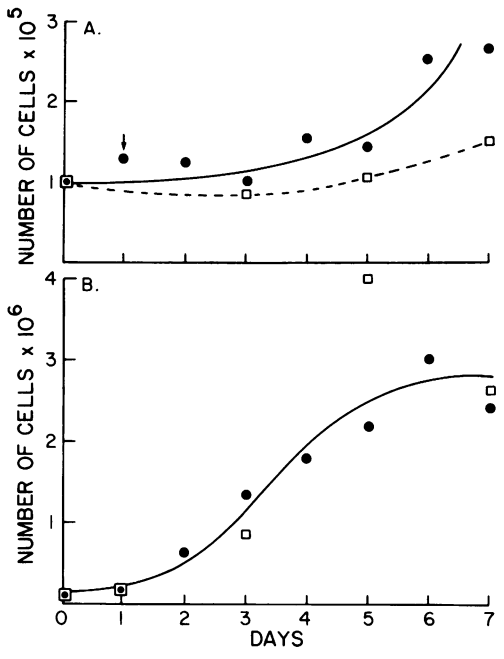


Figure 6—Kinetics of cell growth, at 37 C in 5% CO_2/95% air, in the absence (**A**) or presence (**B**) of a 10% fetal bovine serum supplemented in Dulbecco's minimal essential medium. (●), HT-1080 cells without BABIM exposure; (○), identical cells exposed to 10^{-4} M BABIM for 7 days. The medium was replaced every other day. Each point represents the mean of triplicates. In **A** the correlation coefficients were 0.89 and 0.96 (not significant), and in **B** the correlation coefficients were 0.995 and 0.945 (not significant).

initial nonpassaged cells), by Day 7 the difference between the treated and nontreated groups was 75.2% (403% inhibition by the method of Thorgeirsson), a number in the range of maximal inhibition seen at other treatment dosages at Day 3 (Figure 5).

Type IV Collagen Degrading Activity

In order to verify that, in this model system, BABIM inhibits proteases in general, and proteases which degrade basement membrane proteins in particular, we assessed the HT-1080 fibrosarcoma cell's ability to secrete Type IV collagenase in the presence or absence of BABIM. As is shown in Table 2, at the time of maximum tumor invasion (3 days) BABIM inhibits collagenase activity by 44% ($P < 0.005$). Repeat experiments (data not shown) yielded similarly significant changes in inhibition of Type IV collagenase in the presence of BABIM. This lends further strength to the

Figure 7A—Low-power photomicrograph of HT-1080 cells growing in the lower dermis of the flank of a nude female mouse given an injection at that same site of 2×10^6 cells in 0.1 ml medium vehicle 3 weeks earlier. Note the apparent circumscription of the tumor and the noninvolvement of the overlying epidermis. (H&E; original magnification, ×40). **B**—Metastatic focus of fibrosarcoma cells well seen in the subcapsular sinus (*upper center*) of the right inguinal lymph node draining the primary tumor focus. Tumor cells are admixed with histiocytelike reticular cells in the nodal parenchyma. One island of lymphocytes (*lower right*) is also appreciated. (H&E; original magnification, ×40) **C**—Two foci of intrapulmonary metastases from a BALB/c/NIH-Swiss hybrid mouse given 1×10^6 cells by left intrajugular injection. One focus (*upper*) has an intraalveolar distribution, and the lower one is clearly seen expanding a peribronchial artery. (H&E; original magnification, ×40) **D**—Nest of tumor cells within an alveolar space seen at higher power to demonstrate cellular detail. (H&E; original magnification, ×250)

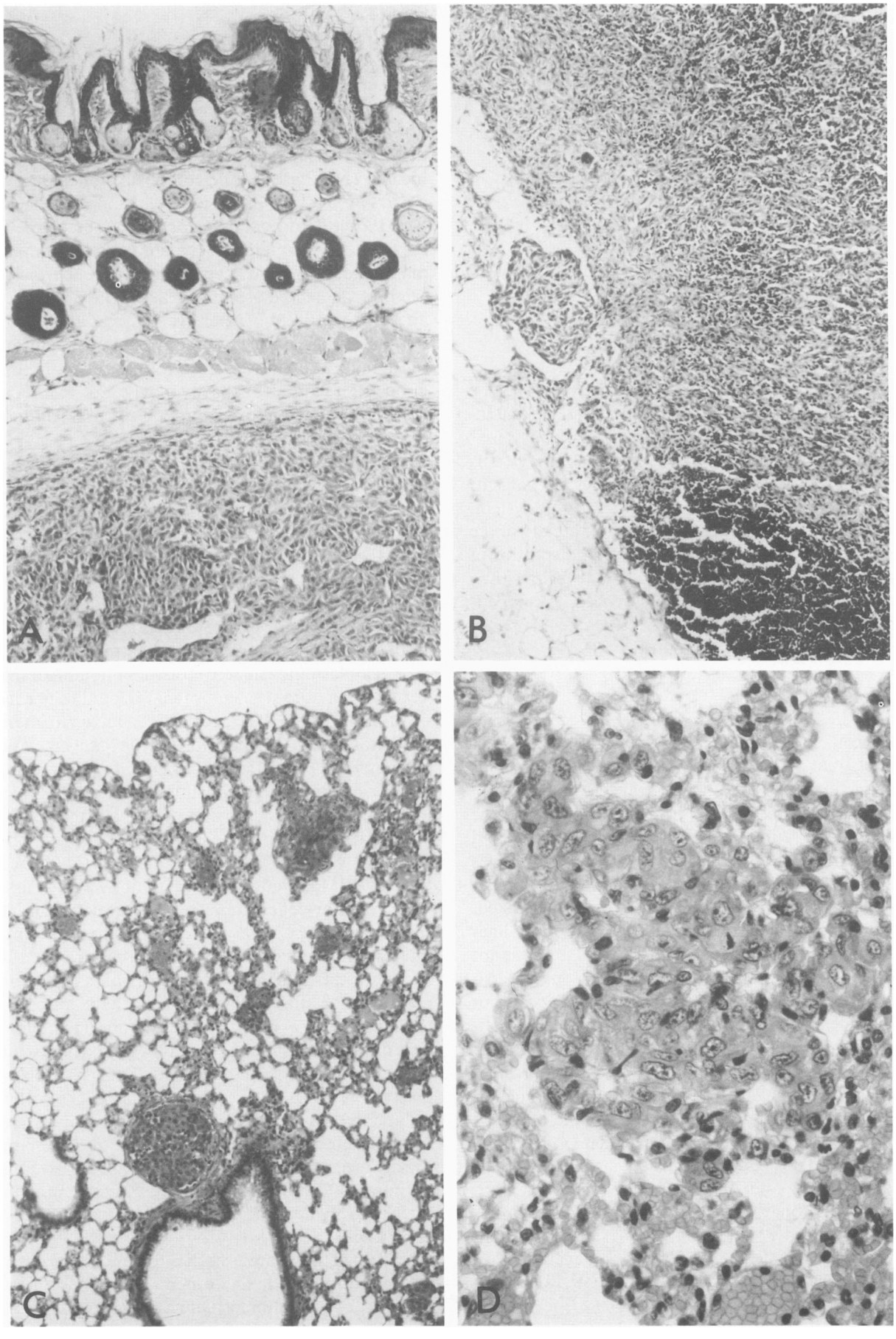

A
B
C
D

Table 2—Type IV Collagenolytic Activity in the Culture Fluid of HT-1080 Cells in the Presence or Absence of BABIM

Treatment*	CPM/10^5 cells/18H	Type IV collagenase activity (mean %)
HT-1080 cells alone	1423.1 ± 44.3†	47.0
HT-1080 + 10^{-4}M BABIM	792.6 ± 87.1	26.1
HT-1080 + bacterial collagenase	3024.7 ± 4.2	100.0
HT-1080 + bacterial collagenase + BABIM	3021.7 ± 4.2	99.9

* See text for details.
† Mean of triplicate samples ± standard deviation.

argument that there is a correlation between Type IV collagenase levels in extracellular supernatants and invasiveness, as monitored by the amnion system, as we[26] and others[50] have previously suggested. Naturally, this does not exclude the possibility that other mechanisms and/or other proteases might operate in this system. Additionally, since BABIM is a potent inhibitor of the active site on trypsin, these types of experiments do not totally exclude the possibility that the trypsin activation of Type IV collagenase is influenced by competition from BABIM. Experiments to exclude this possibility are currently being planned.

Discussion

There is a large amount of experimental evidence that suggests that tumor invasion is a multistep process.[42] Invasion, in turn, is one link in the metastatic cascade.[43] A second series of physiologic events, the fibrinolytic cascade, has also been associated with malignancy and metastasis.[44] Chief among the suspected products is plasminogen activator(s) (PA).[45] PAs are a group of closely related serine proteases which have been linked, in both animal and human primary tumors and cell lines to metastatic ability and tumor invasion. However, not all studies demonstrate such a correlation.[7-9,46] Although the exact role that PA plays in invasion is not entirely clear, it is known that PA modulates the activation of plasminogen to plasmin. Plasmin, in turn, can, in a feedback loop, stimulate PA[47] as well as degrade fibronectin and laminin, the major noncollagenous glycoprotein component of BM, thereby playing a central role in extracellular matrix remodeling.[4,48] Plasmin has also been shown to activate both vertebral collagenase I, II, and III, thereby allowing degradation of interstitial collagens,[49] and latent BM (Type IV) collagenase. This is a potentially critical step in invasion, because levels of activated Type IV collagenase have been directly linked to metastatic potential in malignant cells *in vitro* and *in vivo*.[5,50]

In these studies we have elected to measure the inhibitory effect of BABIM on HT-1080 human fibrosarcoma cells with the use of a quantitative *in vitro* invasion system. This assay, which uses human amnion from normal term placentas, overcomes many previously defined deficiencies in *in vitro* models.[51,52] Advantages include 1) the absence of a complicating nervous, vascular, lymphatic or immune system and 2) the lack of dependence upon nonhuman or artificial matrices. Furthermore, 3) it contains no contaminating host inflammatory cells, 4) is not dependent on the life cycle of the host, 5) can be used to quantify the passed cells, and 6) is relatively inexpensive and readily available. Its greatest advantage, however, is that invasion through the amnion is a relatively accurate predictor of the cell population's invasive capacity *in vivo*, due to its correlation with enzymatic degradation of basement membrane collagen.[25,26,50]

HT-1080 fibrosarcoma cells were chosen in these experiments for a number of critical reasons. This line has been shown to produce BM proteins[53] and both high PA and elevated Type IV collagenase levels in both trypsin activated and nonactivated forms.[6] It has also been shown to have the ability to degrade extracellular matrices in a density-dependent manner.[54] Furthermore, an endothelial cell factor could be shown to inhibit this degrading ability by inhibiting the production of tumor cell plasminogen activators, in a nondirect manner.[55] It therefore seemed prudent to study the effects of BABIM on HT-1080, because it is known to interact at several points along the fibrinolytic pathway, which, when normally functioning, leads to the activation of cellular collagenolytic metalloproteases.

Our data have led us to draw the following inferences and conclusions. First, BABIM, at a concentration of from 10^{-9} to 10^{-3} M, inhibits the ability of HT-1080 cells to invade BM, as judged by the amnion invasion assay. This inhibitory effect at very low concentrations furthermore suggests a very specific receptor ligand type interaction. Second, at the concentrations of from 10^{-5} to 10^{-3} M this inhibition is highly significant for time and treatment effect. Third, this can be quantitated in this sytem equally well by visually counting individual cells or by ^{3}H-labeling cellular DNA. Fourth, this inhibition of invasive ability is maximal at early times after exposure to BM. However, this is not the case for cells derived from tumors passaged in nude mice, where maximal inhibition is seen at the distant time point. Fifth, the mechanism of action of the antiinvasive effect on human fibrosarcoma cells is, at least in part, secondary to the inhibition of the secretion of Type IV collagenase.

We have limited these studies to one facet of tumor invasion, recognizing that other forms of cellular proteinases do have an important role in promoting tumor invasion. The cathepsin-B-like cystein proteinases have

previously been shown to have an impact on a cell population's invasive and metastatic potential.[56,57] These enzymes may cooperate with the collagenases in creating defects in extracellular matrices allowing for cell invasion.[58] They are also probably inhibited by BABIM-like inhibitors. In addition to protein destruction, heparan sulfate degradation of matrices by heparan sulfate endoglycosidase has also been described.[59,60] Furthermore, other events may be critical, such as motility of tumor cells, in invasive processes.[61] Our work does, however, strongly argue that inhibition of esteroproteases, at least in this sarcoma line, will retard invasion to a significant degree. The maximal inhibition of approximately 75%, and no greater, would also support the contention that other factors influence a cell's ability to invade. Recently, evidence has been presented which suggests that there is a threshold level of PA production over which metastatic ability rises dramatically.[62] We are currently testing the ability of BABIM to inhibit metastasis *in vivo*. This, we hope, will give us additional evidence to support or refute this contention.

The human amnion invasion assay serves as an important system for quantitating a highly complex biologic process. The small percentage of cells able to cross the BM is similar to that seen in *in vivo* studies and is most likely a function of tumor cell heterogeneity.[63] By having minimal to no proliferative changes in the presence of inhibition of invasion, this work lends further support to the data suggesting that only protein synthesis, and not DNA synthesis, is needed for invasion.[64,65] We are therefore intrigued by the notion that within a population of neoplastic cells, a subset of cells fully capable of invasion, and by extension, metastasis, may not be dependent upon cell proliferation.

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